The Chloroplast ATP Synthase in

Chlamydomonas reinhardtii

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II. BIOCHEMICAL STUDIES ON ITS BIOGENESIS USING MUTANTS DEFECTIVE IN PHOTOPHOSPHORYLATION*

Claire Lemaire‡ and Francis-André Wollman§

From the Service de Photosynthèse, Institut de Biologie Physico-chimique, 13 rue Pierre et Marie Curie, 75005 Paris, France

We have carried out an analysis of the synthesis, cellular accumulation, and membrane binding of the chloroplast-encoded subunits of the ATP synthase (α, β, ϵ, I, III, and IV) in several mutants of *Chlamydomonas reinhardtii* defective in photophosphorylation. These data gave some insight on the putative genetic lesion in each mutant and allowed some characterization of the assembly and stabilization of the ATP synthase complex in the thylakoid membranes.

Four chloroplast mutants are likely to be altered in chloroplast structural genes coding for coupling factor (CF) 1 subunits β and ϵ and for CF_0 subunits I and IV. A fifth chloroplast mutant and three nuclear mutants were altered in genes regulating either transcription or translation of chloroplast genes coding for CF_1 subunits α and β and CF_0 subunits III and IV.

Evidence is presented (i) for a control of the rate of synthesis of subunit β by subunit α in the absence of ATP synthase assembly and (ii) for an interaction between α and β subunits in the stroma of the chloroplast which protects α subunits from proteolytic degradation.

The role of several chloroplast-encoded subunits of CF_0 and CF_1 in the stabilization of partially assembled ATP synthase is discussed.

We conclude that in the absence of ATP synthase assembly, CF_0 cannot accumulate in the thylakoid membranes, whereas α and β subunits, presumably engaged in soluble CF_1, can accumulate in the stroma of the chloroplast.

Whereas in *Escherichia coli*, the subunits of the ATP synthase complex are encoded by genes clustered in an operon: the unc operon (1), the biogenesis of mitochondria, and chloroplast ATP synthase complexes requires the expression of two genetic systems. Part of the enzyme subunits are encoded in the organelle genome. The others are encoded in the nuclear genome, translated on cytoplasmic ribosomes, and then imported into the organelle. In yeast mitochondria, all the subunits of the extrinsic sector (F_1) and the oligomycin sensitivity conferring protein, which connects F_1 to the intrinsic sector F_0, are encoded in the nucleus, whereas the F_0 subunits are encoded in the mitochondria. The chloroplast ATP synthase presents a more complex organization since both the extrinsic sector, CF_1,[1] and the intrinsic sector, CF_0, comprise subunits encoded in the nuclear and organelle genomes. We have shown in the companion paper (28) that the genetic origin of the chloroplast ATP synthase subunits from the unicellular green algae *Chlamydomonas reinhardtii* is the same as in higher plants: subunits α, β, and ϵ of CF_1 and I, III, and IV of CF_0 are encoded in the chloroplast genome, whereas subunits τ and δ of CF_1 and subunit II of CF_0 are encoded in the nuclear genome.

How nuclear and organelle gene products interact in the membrane assembly of an oligomeric enzyme like the ATP synthase, is a challenging question. Previous reports (2, 3) have shown that the accumulation of all the constitutive subunits of CF_1 in the thylakoid membranes of *C. reinhardtii* is a coordinated process as is the case for PSII (4), PSI (5), and b6/f (6) complexes. Whether synthesis and membrane binding of the ATP synthase subunits also show some coordination remains to be elucidated. Sequential models based on a step by step assembly of membrane-bound subunits of $(C)F_1$ and $(C)F_0$ had been proposed in *E. coli* (7) and *C. reinhardtii* (8). This view has been challenged recently in studies using bacterial and yeast mutants lacking the whole set of genes encoding either F_1 (9) or F_0 subunits (10, 11). Their phenotype is consistent with a preassembly of soluble F_1 followed either by direct membrane binding on preassembled F_0 in *E. coli* (10) or by step by step membrane binding on three unassembled F_0 subunits in yeast mitochondria (11).

The selective arrest of synthesis of the whole set of either CF_0 or CF_1 subunits, in the case of the chloroplast ATP synthase, is presently out of reach owing to the dispersion of their genes on both the nucleus and chloroplast genomes. However, several single mutants defective in photophosphorylation have been genetically characterized in *C. reinhardtii* (2). A biochemical comparative study of such mutants can provide informations on the biogenesis of the chloroplast ATP synthase. We have previously used such an approach to study the mode of assembly, *in vivo*, of another component of the thylakoid membrane, the cytochrome b6/f complex (6). This was achieved by a comparative analysis of the membrane insertion and accumulation of its constitutive subunits in several b6/f mutants.

In this paper, we focus particularly on the chloroplast-encoded subunits of the ATP synthase. We analyze how the absence of specific subunits affects the stability and membrane binding of the other ATP synthase subunits.

[1] The abbreviations used are: CF, coupling factor; SDS, sodium dodecyl sulfate; RBU$_{p2}$Case, ribulose-bisphosphate carboxylase/oxygenase.

EXPERIMENTAL PROCEDURES

Mutant Strains—In this study, we analyzed several mutants from *C. reinhardtii* altered in the ATP synthase complex which are as follows. Chloroplast mutants which were previously described in Ref. 2 and filed in five complementation groups. Strains from each group were selected: FUD50 (group I), FUD16 (group II), FUD17 (group III), FUD18 (group IV), and FUD23 (group V). Nuclear mutants: F54 and ac46 isolated by R. P. Levine (Harvard University, Cambridge, MA) and characterized by Bennoun and Chua (12) and by Levine and Goodenough (13), respectively; thm24 isolated by G. Schmidt (University of Georgia, Athens, GA) and characterized by Piccioni *et al.* (14).

Methods—All strains were grown at 25 °C in Tris acetate/phosphate medium (15) at a light intensity of 300 lux. Cells were harvested in late exponential phase (4.10^6 cells/ml), except for pulse labeling experiments (2.10^6 cells/ml) which were carried out according to Delepelaire (16). Cells were pulse-labeled with 74 kBq/ml [^{14}C]acetate for 45 min or 185 KBq/ml for 5 min (for details, see "Materials and Methods" of the companion paper (28)). Purified thylakoids were prepared according to Chua and Bennoun (17).

ATP synthase activity was characterized *in vivo* by the analysis of the relaxation kinetics, after a saturating flash, of the absorption changes at 515 and 475 nm according to Joliot and Delosme (18).

Denaturing gel electrophoresis, immunoblotting experiments, and autoradiographies were performed as in Ref. 6. Silver staining was performed using the procedure of Gorg *et al.* (19).

The amount of α and β subunits synthetized or associated with the membranes in each strain was estimated by scanning autoradiograms obtained using pulse-labeled cells or thylakoid membranes purified from pulse-labeled cells, respectively. SDS-gel electrophoresis allowed us to estimate the amount of $\alpha + \beta$ subunits:$A_{\alpha+\beta}$ (α and β comigrating in this gel system), whereas urea/SDS-gel electrophoresis allowed us to estimate the amount of β subunit:A_β (α comigrating with a PSII subunit). The amount of α subunit was deduced from the difference $A_{\alpha+\beta} - A_\beta$. The extent of accumulation of the α and β subunits in whole cells and in thylakoid membranes was determined by densitometric scanning of autoradiograms of immunoblots reacted with anti-$\alpha + \beta$ antibodies coupled to radioiodinated protein A.

All scannings were performed using a gel scanner Isco (model 1312; 470-nm filter).

RESULTS

ATP Synthase Activity—We first studied the ATP synthase activity associated with the thylakoid membranes in several mutants previously reported to be defective in photophosphorylation: these included three nuclear mutants, thm24, F54, and ac46, and five chloroplast mutants, FUD50, FUD16, FUD17, FUD18, and FUD23. The ATP synthase activity *in vivo* was estimated by the decay rate of the 475–515 nm electrochromic shift produced by a short actinic flash (20): when controlled by ATP synthase activity, this decay rate increases upon a pre-illumination which activates the ATP synthase but decreases in the presence of ATP synthase inhibitors such as tri-*n*-butyltin as observed in Fig. 1 for the WT strain.

Only did the chloroplast mutant FUD16 show some ATP synthase activity: the decay rate of the electrochromic shift was sensitive to a pre-illumination and was much faster than in the other mutants ($t_{1/2}$ = 450 ms). In addition, it was sensitive to tri-*n*-butyltin as was the WT. The other mutants, represented by the FUD17 strain in Fig. 1, had no detectable ATP synthase activity, as judged from the slow decay of the electrochromic shift even after a pre-illumination. They were characterized by a half-time of the decay phase in the 1350–1800 ms time range (see Fig. 1). The absence of ATP synthase activity in such mutants revealed some ionophoretic properties of tri-*n*-butyltin, which are not yet understood, as can be seen on Fig. 1 where a slight increase in the decay rate of the electrochromic shift in the FUD17 mutant is observed after incubation with tri-*n*-butyltin.

Polypeptide Deficiencies—Thylakoid membranes from these mutants presented the same set of polypeptide deficiencies as that in the FUD50 (see Fig. 2, companion paper (28)), namely subunits α, β, τ, δ, and ϵ of CF_1 and subunits I, II, III, and IV of CF_0, except for the FUD16 and FUD23 mutants in which we detected minor amounts of α, β, and τ subunits after Coomassie Blue staining of the corresponding electrophoretograms (results not shown).

Synthesis of Chloroplast Translates—We then looked for specific alterations in the synthesis of some ATP synthase subunits in these mutants. Such a study required the use of intact cells in which transient polypeptides can be detected after pulse labeling with [^{14}C]acetate, electrophoresis in the presence of SDS, and autoradiography. The high number of nuclear-encoded polypeptides labeled in such experiments prevented us from detecting the subset of ATP synthase subunits of nuclear origin. In contrast, chloroplast translates could be easily distinguished if the pulse labeling was performed in the presence of cycloheximide which inhibits cytoplasmic translation.

Results of such experiments, where the duration of the pulse was 45 min, are shown on Fig. 2. Synthesis of the various chloroplast-encoded subunits in each mutant can be observed on autoradiograms of either two-dimensional gels, for subunits α, β, ϵ, and IV (Fig. 2A), or on urea gels for subunits I and III (Fig. 2B).

Surprisingly, the three nuclear mutants showed altered synthesis of several chloroplast-encoded subunits of the ATP synthase. The ac46 mutant lacked synthesis of the two CF_0 subunits IV (Fig. 2A, *insert 2*) and III (Fig. 2B). The thm24 mutant lacked synthesis of both the α and β subunits of CF_1, whereas the F54 strain displayed no synthesis of the α subunit (see *insert 1* in Fig. 2A).

Four chloroplast mutants were each specifically lacking in the synthesis of one ATP synthase subunit, β in the FUD50 (Fig. 2A, *insert 1*), ϵ in the FUD17 (Fig. 2A, *insert 2*), I in the FUD18 (Fig. 2B), and IV in the FUD 23 (Fig. 2A, *insert 2*). The last chloroplast mutant, FUD16, synthesized all the chloroplast-encoded subunits of the ATP synthase. However, when compared with the WT, it showed increased rates of synthesis of the two CF_1 subunits, α and β (Fig. 2A, *insert 1*) but not of the ϵ subunit (not shown).

We then addressed the question as to whether the absence of synthesis of one chloroplast-encoded ATP synthase subunit would alter the rate of synthesis of some other chloroplast-encoded subunit of the complex. This could be studied conveniently in the case of the α and β subunits of CF_1 which are well detected in the pulse labeling experiments. Fig. 3 shows the autoradiography of the α and β subunits synthesized in whole cells of the WT and of several mutants pulse-labeled for either 5 or 45 min. Independent of the duration of the pulse, the rate of synthesis of the β subunit in the FUD17 mutant lacking the ϵ subunit was severely decreased. The same observation was made in the three mutants lacking either of the CF_0 subunits (not shown). In this type of mutants, the labeled β subunits in 45-min pulse experiments, measured up to about 30% of those synthesized in the WT. Only did the F54 mutant, lacking synthesis of the α subunit, show high rates of synthesis of the β subunit, reaching WT levels in 45-min pulses (Fig. 3B).

In contrast, the rates of synthesis of the α subunit, as judged from the 5-min pulses, were similar in the WT and in the mutants lacking synthesis of any of the other chloroplast-encoded subunits (exemplified by FUD17 *versus* WT in Fig. 3), whereas it dropped down to about 30% of the WT rate in the same mutants after 45 min of pulse labeling (Fig. 3). This indicated that a rapid degradation of most of the newly
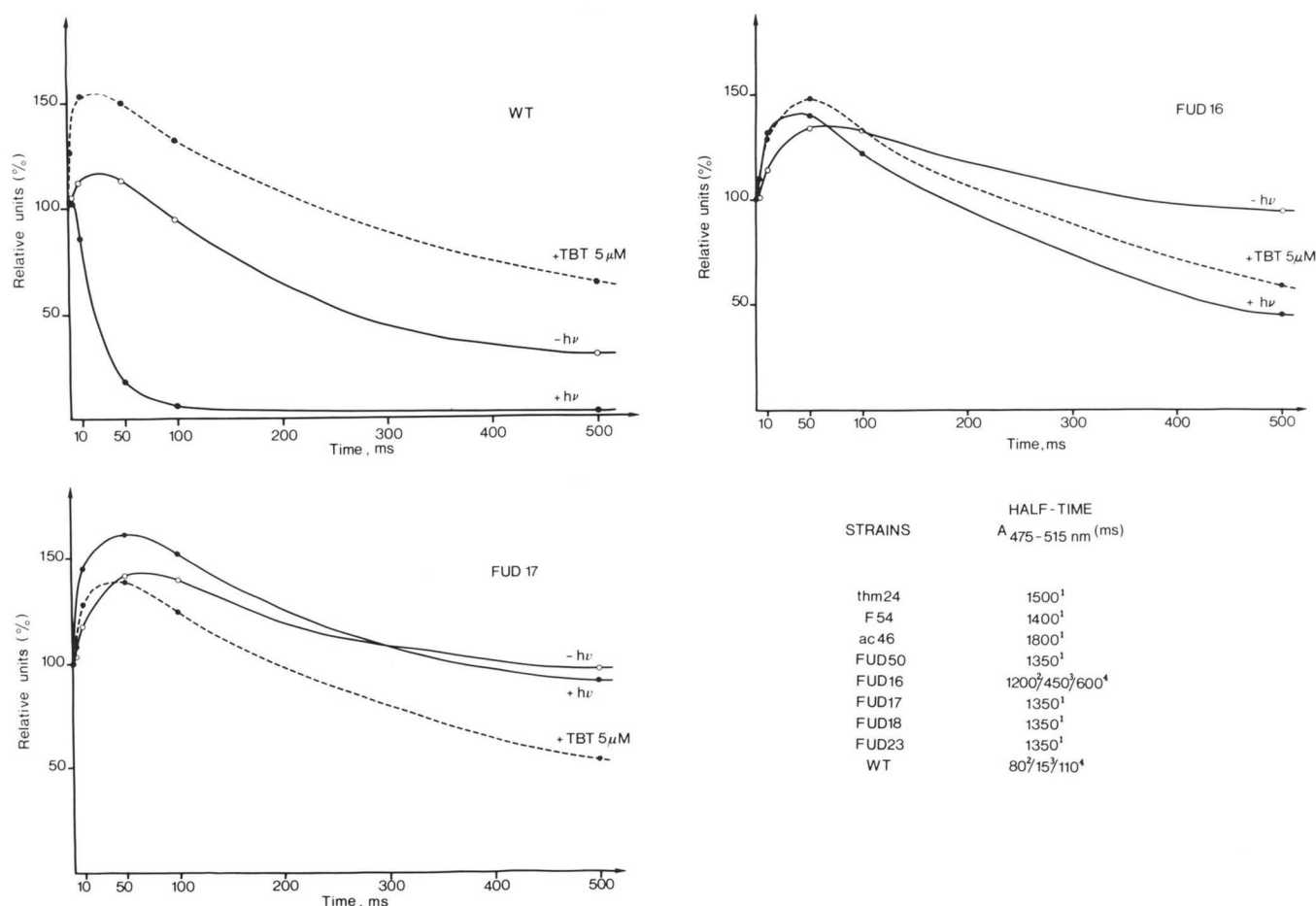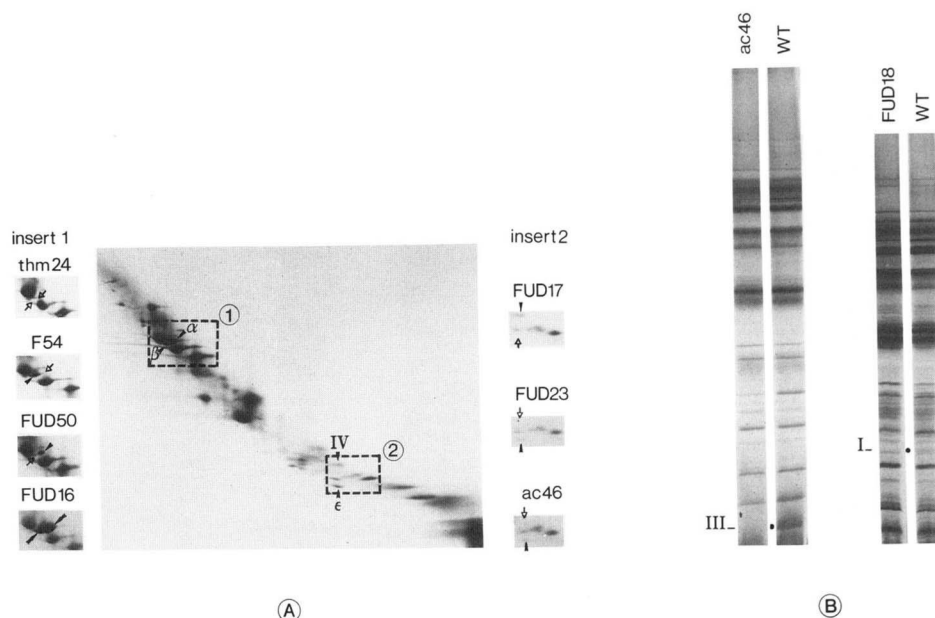
STRAINS	HALF-TIME $A_{475-515\,nm}$ (ms)
thm24	1500[1]
F 54	1400[1]
ac 46	1800[1]
FUD50	1350[1]
FUD16	1200[2]/450[3]/600[4]
FUD17	1350[1]
FUD18	1350[1]
FUD23	1350[1]
WT	80[2]/15[3]/110[4]

FIG. 1. **475–515-nm light-induced absorption changes in WT (fully active ATP synthase), FUD16 (partial ATP synthase activity), and FUD17 (no ATP synthase activity) mutant strains measured with (●) or without (○) a 30-s pre-illumination with broad red light.** *Broken lines* correspond to experiments made in the presence of 5 μM tri-*n*-butyltin which inhibits ATP synthase activity. The results are expressed in relative units referring to the amplitude of the rapid rise phase of the absorption changes detected 300 μs after an actinic flash. Half-time values of the decay phase (corresponding to 50% of the amplitude of the rapid rise phase of the absorption changes) are given for WT and photophosphorylation mutants. *1*, similar in dark-adapted algae or after 30-s pre-illumination; *2*, in dark-adapted algae; *3*, after 30-s pre-illumination; *4*, in the presence of 5 μM tri-*n*-butyltin.

FIG. 2. *A*, autoradiogram of chloroplast translates in WT cells, pulse-labeled 45 min with [14C]acetate in the presence of cycloheximide and viewed after two-dimensional gel electrophoresis. First dimension: 12–18% acrylamide with 8 M urea; Second dimension: 7.5–15% acrylamide gel. *Inserts 1 and 2* correspond, respectively, to the α/β region and to the ε/IV region in various photophosphorylation mutants. *Black* and *white arrows* point, respectively, to the presence and absence of some chloroplast translates. *Double black arrows* point to overproduced chloroplast translates. *B*, same as *A* in one-dimension using urea SDS-polyacrylamide gel electrophoresis. ● indicates the absence of chloroplast translates.
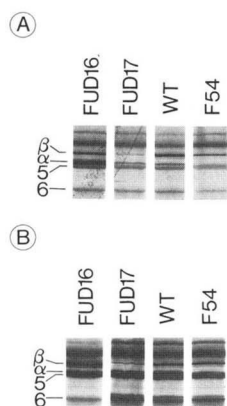
Biogenesis of the Chloroplast ATP Synthase

FIG. 3. **Autoradiograms of chloroplast translates in cells pulse-labeled with [^{14}C]acetate for either 5 (A) or 45 min (B),** in the presence of cycloheximide, viewed after urea/SDS-polyacrylamide gel electrophoresis. Exposure of FUD16 autoradiogram in B is two times shorter in order to preserve satisfactory resolution in the α/β region. Note, (i) the overproduction in the FUD16 mutant *versus* WT of the α subunit only in A and of α and β subunits in B. (ii) The drastic decreased synthesis of subunit β in the FUD17 mutant but not in the F54 mutant. (iii) The heavier labeling of β subunits as compared to α subunits in the WT A but not in B.

FIG. 4. *A*, ϵ region from autoradiograms of chloroplast translates (45-min pulse labeling) bound to the thylakoid membranes in WT and photophosphorylation mutants, viewed after two-dimensional gel electrophoresis (first dimension, 12–18% acrylamide with 8 M urea; second dimension 7.5–15% acrylamide). *Black* and *white arrows* point, respectively, t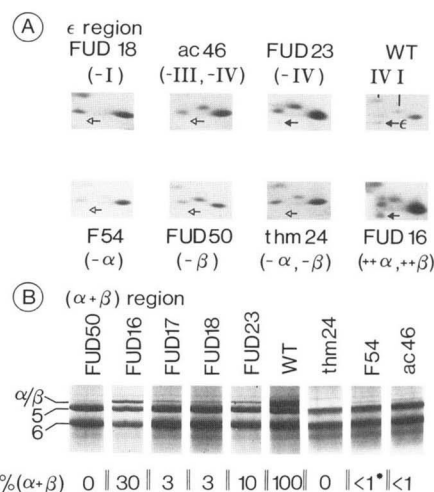o detectable or undetectable subunit ϵ. *B*, $\alpha + \beta$ region from autoradiograms of chloroplast translates (45-min pulse labeling) bound to the thylakoid membranes from WT and photophosphorylation mutants, viewed after SDS-polyacrylamide gel electrophoresis. Amounts of $\alpha + \beta$ subunits, estimated by scanning the autoradiograms, are indicated for each strain and expressed in relative units with respect to the WT strain. *Asterisk*, value corresponding to the amount of subunit β only.

synthesized α subunits occurred in the mutants showing no synthesis of either CF$_1$ (β or ϵ) or CF$_0$ (I, III, or IV) subunits.

The comparison of the rates of synthesis of the α and β subunits in the FUD16 mutant and in the WT shown on Fig. 3 after 45 and 5 min of pulse labeling was particularily intriguing. In the WT, the rate of synthesis of the β subunit in the first 5 min of pulse labeling was higher than that of the α subunit, whereas the two rates tended to be similar after 45 min. Comparison with the level of labeling of PSII subunits 5 or 6 in the two experiments of Fig. 3, shows that it was the rate of synthesis of the β subunit which dropped down in the WT after 45 min of pulse labeling. Whereas overproduction of the two subunits in the FUD16 mutant was visible after 45 min (700% α and 400% β), there was a considerable stimulation of the synthesis of the α subunit only, but not of the β subunit, in the first 5 min of pulse labeling. We hypothesize that the FUD16 mutation results in a block of early posttranslational degradation of some of the β subunits which occurred in the WT between 5 and 45 min of pulse labeling.

Membrane Binding of Chloroplast Translates—We then examined the membrane binding of the transient chloroplast-encoded subunits of the ATP synthase which were still synthesized in the mutants. This was achieved by purification of the mutants thylakoid membranes from the 45-min pulse-labeled cells. The results are shown on Fig. 4 where one can see two-dimensional gel regions containing ϵ, I, and IV (Fig. 4*A*) and the SDS gel regions containing $\alpha + \beta$ (Fig. 4*B*). For a given mutant, the patterns of synthesis and membrane insertion of the three chloroplast-encoded CF$_0$ subunits were identical. This is consistent with an independent co-translational insertion in the thylakoid membranes of these intrinsic subunits. In contrast, the ϵ subunit of CF$_1$ (Fig. 4*A*) was not observed in the thylakoid membrane fractions from the mutants that did not synthesize either α or β of CF$_1$ (F54 and FUD50 mutants) or subunits I or III and IV of CF$_0$ (FUD18 and ac46 mutants). A faint ϵ spot was nevertheless observed in the FUD23 mutant lacking synthesis of subunit IV.

Although the α and β subunits were overproduced in the FUD16 mutant (Fig. 2), they did not bind to the thylakoid membranes to the same extent as in the WT (Fig. 4*B*). We consistently observed that only a small fraction of the α and β subunits synthesized in the mutant cells bound to their

thylakoid membranes (compare for instance, FUD18 *versus* WT in Figs. 2*B* and 4*B*). The absence of membrane insertion of each of the three chloroplast-encoded subunits of CF$_0$ had contrasted effects on the membrane binding of the newly synthesized α and β subunits (Fig. 4*B*): they showed larger membrane binding in the absence of subunit IV (FUD23 mutant) than in the absence of subunit I (FUD18 mutant). Their binding was even lower in the absence of subunit III (ac46 mutant). In the latter case it is, however, difficult to distinguish between a specific effect of the absence of subunit III and the cumulative effect of the absence of both subunits III and IV. The absence of ϵ synthesis in the FUD17 mutant reduced the membrane binding of the α and β subunits to the same extent as the absence of subunit I of CF$_0$.

Last, the comparison of the thylakoid membranes from the F54 and FUD50 mutants on Fig. 4*B* shows that in the absence of α synthesis, membrane binding of newly synthesized β subunits was detectable, whereas the absence of β synthesis prevented significant membrane binding of the α subunit.

Stability of the α and β Subunits—The presence of the α and β subunits in the various mutants was further studied by densitometric scanning of autoradiograms of immunoblots reacted with an anti $\alpha + \beta$ antibody coupled to radioiodinated protein A (Fig. 5). These experiments allowed comparison of both the stability of the α and β subunits synthesized in each strain (Fig. 5*A*) and their extent of binding to the thylakoid membranes with respect to that in the WT (Fig. 5*B*).

The FUD16 mutant cells which showed increased synthesis of the α and β subunits, also showed overaccumulation of these two subunits. The thm24 mutant cells showed no accumulation of the α and β subunits as expected from their absence of synthesis in this strain. Surprisingly, there was no detectable accumulation of the α subunits in the FUD50 mutant cells lacking synthesis of the β subunit, although the α subunits were synthesized in this strain. In contrast the F54 mutant cells, which do not synthesize the α subunit, still

accumulated significant amounts of the β subunit. All mutant cells lacking synthesis of either ϵ (FUD17 mutant) or CF$_0$ subunits (ac46, FUD18 and FUD23 mutants) showed reduced but significant contents in both the α and β subunits.

The immunoblots of the mutant cells showed a heavier labeling on the β subunits than on the α subunits. Part of this uneven labeling was due to the higher titer of our IgG preparation in antibodies against the β subunit than against the α subunit, as can be seen from the uneven labeling of the α and β subunits in the WT membranes which contain $\alpha_3\beta_3$ CF$_1$ (see WT at lower exposure on Fig. 5). However, computation of the respective amounts of α and β subunits relative to that in the WT cells still showed almost a two times excess in β *versus* α subunits in the mutant cells.

Consistent with our pulse labeling study of the membrane binding of newly synthesized α and β subunits was the observation that only a small fraction of the α and β subunits accumulated in the mutants cells, including the FUD16 mutant, were bound to the thylakoid membranes. This is clearly observed in Fig. 5 where the immunoblots of the thylakoid membranes are overexposed as compared to those of intact cells.

The absence of the ϵ subunit (FUD17 mutant) prevented most of the binding of the α subunit but allowed some binding of the β subunit (note the higher contrast in the labeling of these two subunits in the membranes as compared to whole cells of the FUD17 mutant). In the absence of the α subunit (F54 mutant), the β subunit, which accumulated to high levels

in whole cells, could not bind significantly to the thylakoid membranes.

The membrane binding of the α and β subunits differed markedly in the three CF$_0$ mutants: whereas the absence of subunit I (FUD18 mutant) or subunits III and IV (ac46 mutant) prevented significant membrane binding of the α and β subunits, the sole absence of subunit IV (FUD23 mutant) did not prevent binding of the two CF$_1$ subunits. However, binding of the β subunit was much higher than that of the α subunit.

Finally we note that the β-related polypeptide of 70 kDa (see Ref. 28) was present in normal amounts in the cells and in thylakoid membranes of all the mutant strains used in this study.

DISCUSSION

We have compared the patterns of synthesis and membrane binding of the six chloroplast-encoded subunits of the ATP synthase complex: *i.e.* α, β, and ϵ of CF$_1$ and I, III, and IV of CF$_0$, in several chloroplast and nuclear mutants of *C. reinhardtii* defective in photophosphorylation. The data concerning the putative lesion in each mutant are summarized in Table I. We have chosen chloroplast mutant strains from each of the five complementation groups previously identified by Woessner *et al.* (2). These authors have demonstrated that complementation group I, to which belongs the FUD50 mutant, corresponded to lesions in the atpB gene coding for the β subunit. They further hypothesized that there might be a one to one correspondence between these five complementation groups and five of the six structural genes responsible for chloroplast-encoded subunits of the ATP synthase.

Our present study suggests that complementation group III would correspond to mutants altered in the atpE chloroplast gene since the FUD17 mutant, belonging to this group, displayed no synthesis of the ϵ subunit of CF$_1$. Complementation groups IV and V would correspond to mutants altered in the atpF and atpI chloroplast genes, respectively, since we observed no synthesis of subunit I in the FUD18 mutant and no synthesis of subunit IV in the FUD23 mutant.[2] Thus, these complementation groups can be recognized as a tool to map four of the structural genes of the chloroplast-encoded sub-
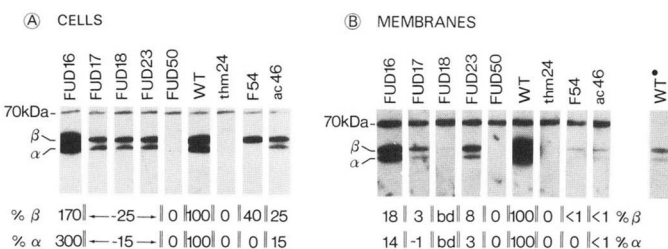

FIG. 5. **Immunoblots using cells (A) and thylakoid membranes (B) from WT and mutants of photophosphorylation after urea/SDS-polyacrylamide gel electrophoresis, probed with anti-$\alpha\beta$ antibodies coupled to radioiodinated protein A.** Exposure time was 24 h in A and above 110 h in B except for WT[•] which was exposed for 2 h. For each strain, the amounts of subunits α and β, expressed in relative units with respect to the WT, were estimated by scanning the autoradiograms.

[2] If the correspondance between subunits I and IV from spinach and *C. reinhardtii* was the opposite of the one we have chosen (see Ref. 28), the altered genes would be atpF in FUD23 and atpI in FUD18.

TABLE I

Strains (origin)	Main characteristics	Putative mutation
FUD50 (chloroplast; group I)	No synthesis of the β subunit	Deletion in the atpB[a] chloroplast gene (2)
FUD16 (chloroplast; group II)	Over production of the α and β subunits	Altered in chloroplast gene regulating the expression of the atpA and atpB chloroplast genes
FUD17 (chloroplast; group III)	No synthesis of subunit ϵ	Altered in the atpE chloroplast gene
FUD18 (chloroplast; group IV)	No synthesis of subunit I	Altered in the atpF chloroplast gene
FUD23 (chloroplast; group V)	No synthesis of subunit IV	Altered in the atpI chloroplast gene
thm24 (N)	No synthesis of α and β subunits	Altered in nuclear gene regulating either transcription or translation of the atpA and atpB chloroplast genes
F54 (N)	No synthesis of subunit α	Altered in nuclear gene regulating either transcription or translation of the atpA chloroplast gene
ac46 (N)	No synthesis of the subunits III and IV	Altered in nuclear gene regulating either transcription or translation of the atpH and atpI chloroplast genes

[a] atpA, gene encoding for the α subunit of CF$_1$; atpB, gene encoding for the β subunit of CF$_1$; atpE, gene encoding for the ϵ subunit of CF$_1$; atpF, gene encoding for the subunit I of CF$_0$; atpH, gene encoding for the subunit III of CF$_0$; atpI, gene encoding for the subunit IV of CF$_0$.

units of the ATP synthase on the plastid chromosome. Looking for mutants altered in the genes encoding for subunits α and III would be of interest to complete the mapping.

The FUD16 mutant, belonging to complementation group II, may be altered in a gene regulating the assembly of ATP synthase since it showed no lack in synthesis of any of the chloroplast-encoded subunits of the complex, had detectable chloroplast ATP synthase activity, but overaccumulated the α and β subunits. Alternatively it could be altered in the expression of the atpA gene only, since the increased synthesis of the α subunit viewed in the 5-min pulse labeling experiments may account for the overaccumulation of both the α and β subunits as will be discussed below.

The three nuclear mutants, thm24, F54, and ac46, were altered in nuclear genes regulating either transcription or translation of chloroplast genes. It should be noted that such nuclear control of chloroplast gene expression has been previously reported in *C. reinhardtii* for the psbD gene of PSII (21) and for the psaA gene of PSI (22). The cases of the thm24 and ac46 nuclear mutants are particularly intriguing since they both show a block in the synthesis of two chloroplast-encoded subunits, α and β, and III and IV, respectively. Neither the genes of the α and β subunits nor those of subunits III and IV (respectively I, see Footnote 2) are located on neighboring regions of the plastid chromosome (23) and therefore should not be co-transcribed. Further analysis of the chloroplast mRNAs in these mutants will be required to understand at which level these nuclear genes act.

In any of the CF_0 mutants, (FUD18, FUD23, and ac46) as in the CF_1 mutant lacking the ϵ subunit (FUD17), most of the α and β subunits did not bind to the thylakoid membranes and were therefore localized in the stroma of the chloroplast. We observed a close proportionality between the extent of cellular accumulation of subunits α and β (about 25% and 15% of the WT levels for subunits β and α, respectively, as detected on immunoblots) and their rate of synthesis (about 30% of the WT levels for both subunits as measured in 45-min pulse labeling studies). This indicated a surprising stability of the two subunits when not assembled in a membrane bound ATP synthase complex, which contrasts with the efficient proteolytic degradation of the unassembled $RBU_{p2}Case$ subunits in the stroma of the chloroplast (24). It has, however, been demonstrated that F_1 subunits assemble in a soluble form in the absence of F_0 both in *E. coli* and in yeast mitochondria (10, 11). In addition, soluble $\alpha\beta\tau$ complexes were identified in the absence of ϵ synthesis in *E. coli* (10), a situation which may prevail in the FUD17 mutant presently described. It is then likely that the α and β subunits are similarly assembled in soluble complexes, thus protected from denaturation and proteolytic degradation in the stroma of the chloroplast of the various mutants we have analyzed. Further evidence for the occurrence of $\alpha\beta$ interactions in the stroma of the chloroplast in the absence of ATP synthase assembly came from the comparison of the contents in α and β subunits in two mutants lacking either α (F54) or β (FUD50) synthesis: β subunits were stable in the absence of the α subunits (detected both in pulse labeling studies and on immunoblots), whereas the latter did not accumulate (detected in pulse labeling studies but lost on immunoblots) in the absence the β subunits. In the latter case we did not observe new electrophoretic bands or stainable material on the top of the gels which would be indicative of α denaturation either by self-aggregation or by complex formation with ubiquitin-like proteins. Therefore subunit α was most likely lost by rapid proteolytic degradation rather than by denaturation. We conclude that an interaction with the β subunits is

required to protect the α subunits from proteolytic degradation in the stroma of the chloroplast. The stromal stability of the β subunits in the absence of the α subunits is intriguing but may be due to persisting interactions with other CF_1 subunits.

Two additional sets of interaction between the α and β subunits arose from our comparative pulse labeling studies on the WT and mutant strains. The rate of synthesis of the β subunit was reduced to about 30% of that in the WT in all the ATP synthase mutants, but in the FUD16 mutant showing some ATP synthase activity and in the F54 mutant lacking the α subunit. This suggested a restricted translation of the β subunit in the absence of assembled ATP synthases which would be relieved when the α subunit is not synthesized. Such a control could occur through an interaction between the nascent β polypeptide, or the atpB mRNA-ribosome complex, and an α subunit, leading to decreased translation of the β subunit only when the α subunit is not engaged in the assembly of an ATP synthase complex. Conversely, the rate of synthesis of the β subunit was by far larger than that of the α subunit during a 5-min pulse labeling of the WT cells. The resulting excess of β subunits was, however, rapidly degraded since 45-min pulses showed the same labeling on the α and β subunits in the WT. This degradation was not observed in the FUD16 mutant in which α synthesis was stimulated. This suggests that the newly synthesized β subunits are stabilized by an interaction with the newly synthesized α subunits in these two strains which show ATP synthase activity.

This latter interpretation appears conflicting with the stromal stability of the β subunits in the absence of synthesis of subunit α. However, it should be noted that one situation relates to the metabolism of the two CF_1 subunits when active chloroplast ATP synthases are assembled (FUD16 and WT), whereas the stromal stability of β was observed in mutant strains lacking such ATP synthase complexes. As a consequence, the energetic of the chloroplast is deeply modified in the light, with ATP generated in one case and high thylakoid membrane potential accumulated in the other case. Translatable mRNAs for the α and β subunits have been found both on free and membrane-bound ribosomes in pea and *C. reinhardtii* (8, 25). In addition, ribosome binding to the thylakoid membranes is light dependent in the WT of *C. reinhardtii* (26). We suggest that, in the light, the presence of ATP synthase complexes in the thylakoid membrane controls synthesis of the β subunits on thylakoid-bound ribosomes, where non-stoichiometric amounts of β subunits would be available to a membrane-associated protease. In contrast, β and α subunits would rather be synthesized on free ribosomes in mutants lacking ATP synthase complexes, thus placed in a different environment where isolated α subunits would be degraded by soluble proteases unless complexed with β subunits. These hypotheses are currently under investigation.

Comparison of the membrane contents in ATP synthase subunits in the various mutants brought some insights in the possible mode of assembly and in the stabilization of the chloroplast-encoded subunits of the ATP synthase in the thylakoid membranes. The chloroplast-encoded CF_0 subunits that were still synthesized in the various CF_0 and CF_1 mutants were membrane inserted, as viewed by ^{14}C-pulse labeling, but did not accumulate since they were lacking in Coomassie Blue or silver-stained electrophoretograms. The absence of active CF_0 in all cases was further supported by the long lifetime of the membrane potential generated after one actinic flash, which shows that there was no dissipation of the potential through a proton channel. Thus, we conclude that there is a concerted accumulation of the CF_0 subunits in the thylakoid

membranes of *C. reinhardtii*, which depends *in vivo* on their assembly with CF_1. In this respect the assembly of the chloroplast ATP synthase seems to differ widely from that in bacteria (9) where accumulation of F_0 subunits and assembly of a functional proton pore has been reported to occur in the total absence of F_1. Such a difference may be related to the distribution of the structural genes of CF_1 and CF_0 which are mixed on two different genomes, in the nucleus and in the chloroplast. An efficient post-translational disposal of CF_0 subunits, when unassembled with CF_1, may be required to maintain proton impermeability of the thylakoid membranes during ATP synthase biogenesis. However, such a stabilization of CF_0 by assembly with CF_1 does not preclude the possibility that transient CF_0 subunits would assemble before interacting with CF_1.

Although our experiments did not allow a direct study of the assembly of the CF_0 subunits in the membrane, some insight into their respective roles in the assembly of the ATP synthase was obtained by comparing the membrane binding of subunits α and β in the CF_0 mutants. The very low amount of membrane-bound CF_1 subunits in the mutants is consistent with the drastic decrease in the number of CF_0 binding sites for CF_1 subunits in the thylakoid membranes as discussed above.

Role of Subunit IV—We showed that significant binding of the α and β subunits (5–10% of that in the WT) occurred in the absence of subunit IV (FUD23 mutant). Membrane binding of the τ and ϵ subunits could also be detected in the latter case. It is then likely that CF_1 can bind on CF_0 sectors depleted of subunit IV. Such partly assembled $CF_1 \cdot CF_0$ complexes would accumulate in the thylakoid membranes of the mutant up to 10% of the WT level, with CF_0 subunits still remaining below detection on gels by conventional staining procedures. However, the presence of subunit IV further stabilizes the complexes in the membranes and is required for ATP synthase activity as judged from the longlife time of membrane potential in the FUD23 mutant.

Role of Subunit III—Only trace amounts of membrane-bound α and β subunits were observed when subunit III was lacking together with subunit IV (ac46 mutant). This observation is consistent with the major part played by subunit III in the formation of CF_0 (27) and supports the view that subunit III is required for membrane binding of CF_1, as suggested in yeast mitochondria for the homologous F_0 subunit 9 (11).

Role of Subunit I—In contrast with the similarity in relative amounts of membrane-bound α and β subunits deduced from pulse-labeling experiments and from the immunoblots in most of the CF_1 and CF_0 mutant, only transient binding of some of the newly synthesized α and β subunits occurred in the absence of subunit I (membrane-bound α and β subunits were detected in pulse-labeling experiments in the FUD18 mutant but lost on immunoblots). A possible explanation for such transient binding in the absence of subunit I is that the lifetime of newly formed binding sites for subunits α and β on the membrane, probably subunit III, is too short to interact with soluble CF_1 accumulated in the stroma, but long enough to interact with newly synthesized α and β subunits. This could be the case if, for instance, diffusion of CF_1 from the stroma to the membranes was much slower than that of the newly synthesized, unassembled α and β subunits. Subunit I would then behave like a subunit regulating the stability of $CF_1 \cdot CF_0$ complexes in the membranes, by protecting α and β binding sites (presumably subunit III) from early proteolytic degradation.

The Chloroplast-encoded CF_1 Subunits—In the two cases

where significant binding of the α and β subunits was detected, in the FUD17 and FUD23 mutants, we note that there was about a 3-fold excess of binding of subunit β over that of subunit α. The origin of this non-stoichiometric binding is unclear: either the full complement of subunit β binds to CF_0 before the binding of the first subunit α or β homodimers compete efficiently with $\alpha\beta$ heterodimers in the binding to CF_0 in these two mutants. Whereas, membrane binding of the β subunit was severely hampered in the absence of the α subunit (F54 mutant), the absence of the ϵ subunit (FUD17 mutant) allowed some membrane binding of the α and β subunits. Although $(C)F_1$ preassembly may occur in a soluble form, the above observation is consistent with an assembly pathway of the ATP synthase which does not require preassembly of CF_1. Binding of subunits α and β to CF_0 could precede ϵ binding, which in turn would stabilize CF_0 in the thylakoid membranes. It remains, however, difficult to assess how closely oligomeric associations of ATP synthase subunits detected in photophosphorylation mutants reflect genuine steps in the assembly of the active enzyme. In particular, as suggested by the differential stability of the β subunits in the presence or absence of active ATP synthase, the possible translation of the α and β subunits on thylakoid-bound or free ribosomes in the WT or photophosphorylation mutants, respectively, may control kinetically the transient formation of different oligomeric complexes of ATP synthase subunits in the two situations.

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